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Evidence that the deep keratin filament systems of the *Xenopus* embryo act to ensure normal gastrulation

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ABSTRACT **To study the role of keratin filaments in *Xenopus* development, fertilized eggs were injected with anti-keratin monoclonal antibodies. The anti-keratin monoclonal antibodies AE1 and AE3 induce abnormal gastrulation; in the most severely affected embryos gastrulation fails completely. In contrast, embryos injected with the anti-keratin antibody 1h5 develop normally. Immunocytochemical data indicate that injected 1h5 binds to the dense superficial keratin filament system of the embryo but not to the deeper keratin filament networks of ectodermal and subectodermal cells. Injected AE1 and AE3 do not bind to the superficial keratin system but appear to interact preferentially with the deep keratin filament systems of the embryo. We conclude that the superficial keratin filament system is not involved in the process of gastrulation *per se* but may protect the embryo from mechanical damage. On the other hand, our results suggest that the integrity of the deeper keratin filament systems is required for the mechanical integration of the morphogenetic movements that underlie gastrulation in *Xenopus*.**

Intermediate filaments (IFs) are a major structural component of metazoan cells (1). There are many different IF proteins and different IF proteins are expressed in different cell types (2). Perhaps the most dramatic example of the diversity in IF composition is found in the keratins, which are characteristic of epithelial cells. There are now >30 keratin genes identified in the human and each epithelial cell type expresses a characteristic complement of keratins (3). On the basis of their resistance to mechanical stress and their ability to transmit tension through desmosomal junctions, it has long been assumed that keratin-type IFs act as mechanical stabilizers of cells and tissues (see ref. 4). Recent work in both mice and humans supports this hypothesis (see ref. 5). However, outside the mammalian epidermis very little is known about keratin filament function. We have therefore turned to the anuran amphibian *Xenopus laevis* as a readily accessible model system for studying the role of keratin filaments not only in epidermal-like tissues but also in the morphogenetically active epithelial tissues.

At fertilization, the keratin filament system of the *Xenopus* egg is disassembled and exists as a heterogeneous population of soluble oligomers (6). Fertilization initiates keratin reassembly (7). By the end of the first cell cycle, the embryo has a polarly asymmetric superficial keratin filament system consisting of a dense network of keratin filament cables in the vegetal hemisphere and fine "disorganized" keratin filaments in the animal hemisphere (7). In addition to this superficial keratin filament system, the embryo also contains an organizationally distinct "deep" keratin filament system consisting of fine filaments (ref. 7 and see below). The organizational difference between the superficial and deep keratin filament systems does not appear to be due to their composition. Keratin filaments are obligate hetereopolymers of type I and type II keratins (3). Given that only a single type II keratin has been identified in the early embryo (8), it should be present in all filaments. The two type I keratins present (9) are also expected to readily copolymerize with the type II keratin and each other (3). Presumably, factors other than the keratins themselves are responsible for regional differences in keratin filament organization.

Desmosomes, linking the deeper keratin filament networks of individual blastomeres, appear well before gastrulation and become increasingly prominent as development continues (10). Until the midblastula transition, when embryonic transcription begins (11), the embryonic keratin system is composed of only the three keratins that are present in the egg. From the midblastula transition (stage 8.5—6.5 h at room temperature) to the beginning of gastrulation (stage 10—9 h after fertilization) (12), new transcription begins in earnest and new keratins appear. These are primarily epidermal-type keratins (13, 14) and they appear to accumulate preferentially in the embryo's superficial keratin filament system (15). Nonkeratin intermediate filament proteins do not appear until well after the end of gastrulation (see ref. 16).

The intracellular injection of anti-keratin monoclonal antibodies (mAbs) has been shown to specifically disrupt keratin organization in both cultured cells (17) and in the early mouse embryo (18). The speed of development in *Xenopus* and absence of embryonic growth until late into development make it feasible to use antibody injection to study the roles of keratins throughout the period of rapid cell division and well into the morphogenetic processes of gastrulation and the beginnings of neurulation. We report here the effects of four anti-keratin mAbs injected into the fertilized egg. We find evidence that the superficial keratin filament system has no active morphogenetic role in early development. In contrast, the integrity of the embryo's deeper keratin filament systems appears to be critical to ensuring normal gastrulation in *Xenopus*. A preliminary report of these results has appeared (19).

MATERIALS AND METHODS

mAbs and Western Blot Analysis. The monoclonal anti-keratin mAbs AE1 and AE3 (20) were generously supplied by T.-T. Sun (New York University Medical Center); anti-IFA (21) was obtained from the American Type Culture Collection; 1h5 and E7 (6, 7) are available through the Developmental Biology Hybridoma Bank (Ames, IA). Antibodies were purified from tissue culture supernatants by using the high-salt protein A-Sepharose method described by Harlow and Lane (22). Antibodies were concentrated to using a Centricon-30 microconcentrator (Amicon) and washed five times with antibody-injection buffer (88 mM NaCl/1 mM KCl/15 mM Tris·HCl, pH 7.5); antibody concentrations were measured using a Coomassie blue binding assay (Pierce) with

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Abbreviations: IF, intermediate filament; PYP, protruding yolk plug; mAb, monoclonal antibody.

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bovine serum albumin as a standard. For each blot, five stage-13 embryos were solubilized and analyzed by isoelectric focusing and polyacrylamide gel electrophoresis as described (6).

Injection of Fertilized Eggs. Eggs were obtained from hormonally primed females, fertilized, and dejellied (7). Fertilized eggs, judged by the characteristic cortical contraction and the appearance of a sperm entry point, were transferred to full-strength Ringer's supplemented with 5% (wt/vol) Ficoll (400 kDa) for injection. Eggs were injected with 20 nl of antibody solution in the animal hemisphere within 36 min of fertilization (first cleavage occurred ≈90 min after fertilization) using a timed-pressure device designed by Alberto Domingo (University of Colorado, Boulder). After injection, embryos were transferred to 20% Ringer's/5% Ficoll and held at 16–18°C until stage 7/8 when they were transferred to 20% Ringer's and maintained at 16–18°C until stage 10. The embryos were then transferred to room temperature (22–24°C) and followed through the end of gastrulation (stage 13/14; stages determined according to ref. 12).

Analysis of Antibody Effects. To analyze antibody-injected embryos, we used two methods. In both cases, embryos were analyzed when buffer- or control-antibody-injected embryos had reached stage 13. In the first method, embryos were examined and classed into one of five categories: normal, small "protruding yolk plug" (PYP), medium PYP, large PYP, or complete exogastrula. In the second method, embryos were videotaped, digitized, and analyzed using the IMAGE Version 1.42 program written by Wayne Rasband (National Institutes of Health Resource Service Branch, Bethesda, MD) to determine the area of the exposed yolk plug, as viewed from the vegetal pole.

Immunocytochemical Analysis. Embryos were fixed and stained in whole mount as described (23). Injected antibody was visualized using either peroxidase- or fluorescein-conjugated anti-mouse immunoglobulin antibodies. Staining with 1h5 and rhodamine-conjugated secondary antibody was used to visualize the effects of injected antibodies on the organization of the superficial keratin filament system.

RESULTS

In *Xenopus*, gastrulation begins ≈10 h after fertilization and is complete by 15 h (12). Antibodies injected into the fertilized egg during the first third of the first cell cycle come to be evenly distributed in the embryo by the time of first cleavage (Fig. 1 *A* and *B*). That substantial amounts of the injected antibody remain intact well past the beginning of gastrulation can be demonstrated by Western blot analysis of single injected embryos (data not shown). For these studies, we characterized four anti-keratin mAbs, AE1, AE3, 1h5, and anti-IFA. All are mouse IgG-type antibodies and all label the keratin filament system of fixed and bleached embryos (see below). On the basis of two-dimensional gel blot analyses of gastrula-stage embryos, AE1 and AE3 appear to react specifically with keratins (Fig. 2 *A* and *B*). In contrast to its pattern of specificity in mammalian cells (20), AE1 reacts with both type I and type II keratins. 1h5 reacts with *Xenopus* type II keratins and with a pair of soluble polypeptides present in the *Xenopus* oocyte and embryo (6, 9); anti-IFA reacts with the type II keratins and may also react with lamins (Fig. 2 *C* and *D*) (24).

Injection can induce transitory nonspecific effects in the injected cell (17). In *Xenopus*, injection of buffer alone often results in a delay in the start of gastrulation of ≈1 h, when compared to uninjected embryos. To control for these effects, eggs were injected with either buffer alone or with the anti-β-tubulin mAb E7. E7, also a mouse IgG, can be injected into eggs at concentrations of 20 mg/ml and higher without discernible affects on normal cellular or developmental behavior (Fig. 3 *A* and *B*). As the anti-keratin antibodies used, mAb E7 persists within the embryo in an apparently intact form, as judged by the electrophoretic migration of E7 heavy and light chains on SDS gels (data not shown).

The effects of injected antibodies on development were analyzed at two stages: prior to the beginning of gastrulation (stage 7/8) and at the end of gastrulation (stage 13/14). At stage 7/8, embryos were examined to determine whether they were cleaving normally; of the antibodies tested only

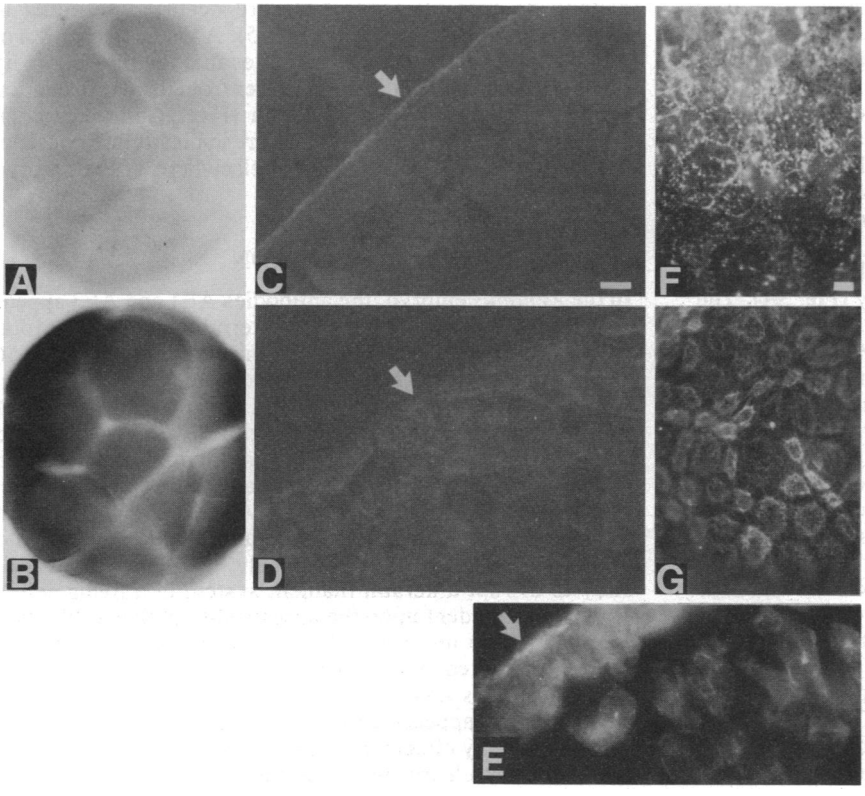

FIG. 1. To analyze the distribution of injected antibodies, embryos were stained in whole mount (23) with peroxidase-conjugated secondary antibody at the 16-cell stage (*A* and *B*). There is little nonspecific staining of uninjected embryos (*A*); the entire embryo appears stained when the fertilized egg is injected with antibody AE3 (*B*). To visualize the distribution of antibody at the end of gastrulation, embryos were stained in whole mount (16) with fluorescein-conjugated secondary antibody, embedded in LR White resin, and sectioned. (*C*) A 1h5-injected embryo. (*D*) An AE3-injected embryo. For 1h5, the injected antibody is confined to the superficial system (arrow), whereas AE3 is excluded from this cortical region and is found associated with deeper structures. The epitope recognized by AE3 is present in both superficial and deeper systems, as can be seen in the section (*E*) of an embryo fixed, bleached, and stained in whole mount with AE3. Cortical whole-mount immunocytochemistry (6) of a 1h5-injected embryo stained with 1h5 (*F*) indicates that the superficial keratin filament system has been disrupted; in an uninjected (*G*) or AE3-injected (data not shown) embryo, the superficial keratin filament system remains intact. (Bars: in *A*, 100 μm for *A* and *B*; in *C*, 10 μm for *C–E*; in *F*, 10 μm for *F* and *G*.).

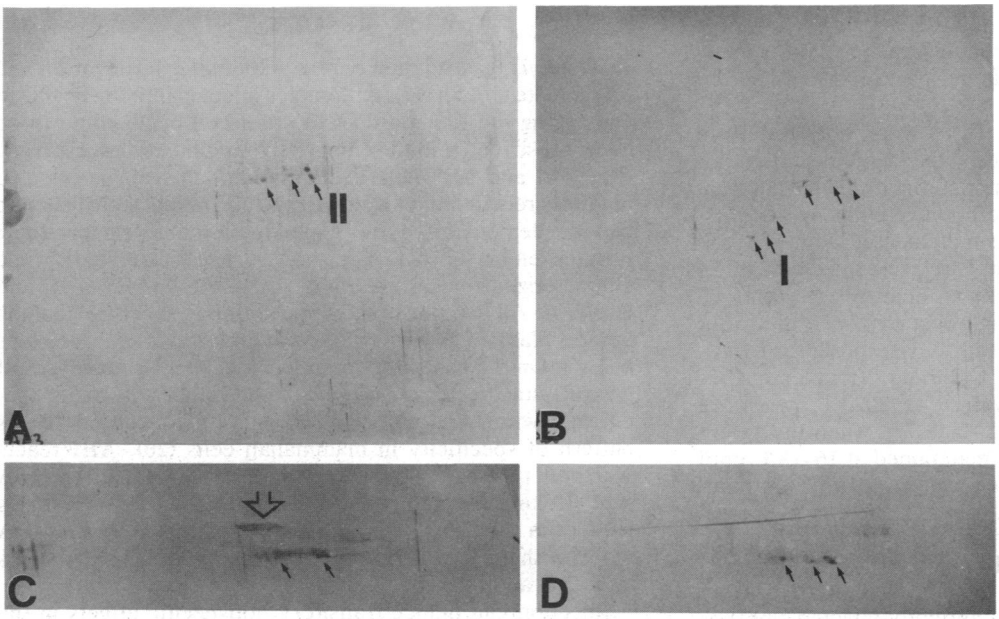

FIG. 2. To assay the specificity of the antibodies used in this study, *Xenopus* embryos were solubilized and analyzed by two-dimensional gel electrophoresis/Western blot. AE3 (*A*) reacts specifically with type II keratins (marked by arrows and II); AE1 (*B*) reacts with these same keratins and with the type I keratins (marked by arrows and I); 1h5 (*C*) and anti-IFA (*D*) react with the type II keratins (small arrows). 1h5 also reacts with larger unidentified polypeptides (open arrow in *C*).

anti-IFA consistently induced defects at this stage. These defects involved irregular cell cleavage and apparent cell death (data not shown). Because keratin filaments do not appear to be directly involved in cell division (4), this effect of anti-IFA is unlikely to be due to the disruption of keratin filaments; more likely this cellular defect may be due to the interaction of anti-IFA with other nonkeratin polypeptides in the embryo. Although anti-IFA does produce defects in gastrulation, the cellular defects it induces complicate the interpretation of these experiments and so are not considered further here. AE1, AE3, and 1h5 have no effect on embryos through the blastula stage of development.

By stage 13, gastrulation is complete, and neurulation has begun. In normal embryos, the yolk plug has been completely internalized at this stage (i.e., area of exposed yolk plug is 0). Embryos injected with >60–90 ng of AE1 or AE3 antibody showed a consistently greater gastrulation failure rate than their uninjected or buffer-, E7-, or 1h5-injected siblings (Fig. 3 *A* and *B*). The severity of the gastrulation defects observed increased as more AE1 or AE3 antibody was introduced (Fig. 3*B*). Given that the *Xenopus* egg has a diameter of 1200 mm and that approximately one-half of its cytoplasmic volume is occupied by yolk platelets, the final cytoplasmic antibody concentrations in the experiment illustrated in Fig. 3*B* are between 0.14 and 0.43 mg/ml for AE1 and between 0.20 and 0.62 mg/ml for AE3—i.e., somewhat lower than those used to disrupt keratin filament organization in cultured cells (17).

Eggs injected with 1h5 gastrulate normally (Fig. 3*A*). The lack of a 1h5 effect on *Xenopus* gastrulation is not due to its rapid degradation within the embryo; Western blot analysis of antibody-injected embryos reveals that 1h5 antibody persists as well as other antibodies (data not shown). Immunocytochemical analysis revealed that injected 1h5 antibody bound to the superficial keratin filament system (Fig. 1*C*); little if any 1h5 antibody was seen in the deeper regions of the embryo. Cortical immunocytochemistry indicates that injected 1h5 antibody can disrupt the integrity of the superficial keratin filament system (Fig. 1 *F* and *G*). In contrast, AE1 and AE3 did not bind to the superficial keratin filament system at all but appeared to bind to structures in the deeper regions (Fig. 1*D*). The failure of AE1 and AE3 to react with the superficial keratin filament system is not due to the absence of their epitopes in these keratin filaments—both antibodies stain both the superficial and deeper keratin filament systems after fixation and bleaching (Fig. 1*E*), implying that their epitopes, although present in the superficial keratin system, are normally inaccessible to AE1/AE3 antibodies in the living embryo.

A complete analysis of the gastrulation defect induced by the anti-keratin antibodies AE1 and AE3 is beyond our scope here but a brief description is clearly warranted. In both AE1- and AE3-injected embryos, embryonic development appeared normal up to the beginning of gastrulation. The dorsal lip formed and its medio-lateral extension appeared normal. The extension of the animal hemisphere over the yolk plug, however, was clearly abnormal (Fig. 3*C*). By the time that uninjected and buffer- or E7 antibody-injected sibling embryos had completed gastrulation (Fig. 3*C*, frames 6–8) and were beginning neurulation (stage 13/14), most AE1/AE3-injected embryos had substantially exposed yolk plugs (Fig. 3*C*, frame 1); these yolk plugs remained exposed through subsequent development. Often gastrulation failed completely, no true blastoporal closure occurred resulting in exogastrulation (Fig. 3*C*, frames 2–5). A visual inspection of AE1/AE3-injected embryos indicates that the blastopore lip often remained at its original diameter from its first formation through to the end of gastrulation (Fig. 3*C*, frames 2–5). Whether this reflects a specific failure in blastopore constriction or is the product of a defect elsewhere in the embryo remains to be determined.

DISCUSSION

In the *Xenopus* embryo, the antibodies AE1 and AE3 appear specific for keratins, based on Western blot analysis of total embryonic protein (Fig. 2). When injected into *Xenopus* A6 cells, which express the same three keratins found in the egg, AE3 does disrupt keratin filament organization (data not shown). In most cases, however, neither AE1 nor AE3 bind to A6 cell keratin filaments, even though these keratin filaments can be stained with these antibodies after fixation. The behavior of these antibodies in A6 cells is similar to that seen previously with other anti-keratin mAbs, injected into cultured epithelial cells (17)—namely, the ability of an antibody to disrupt a keratin filament system in a living cell is critically dependent upon the accessibility of that antibody's epitope. Within any particular cell, some keratin filaments can be disrupted and others are unaffected by injected antibodies. This ability to distinguish between keratin filament systems appears to underlie the differences in anti-keratin antibody effects in *Xenopus*. Whole-mount immunocytochemistry clearly indicates that cytoplasmic AE1 and

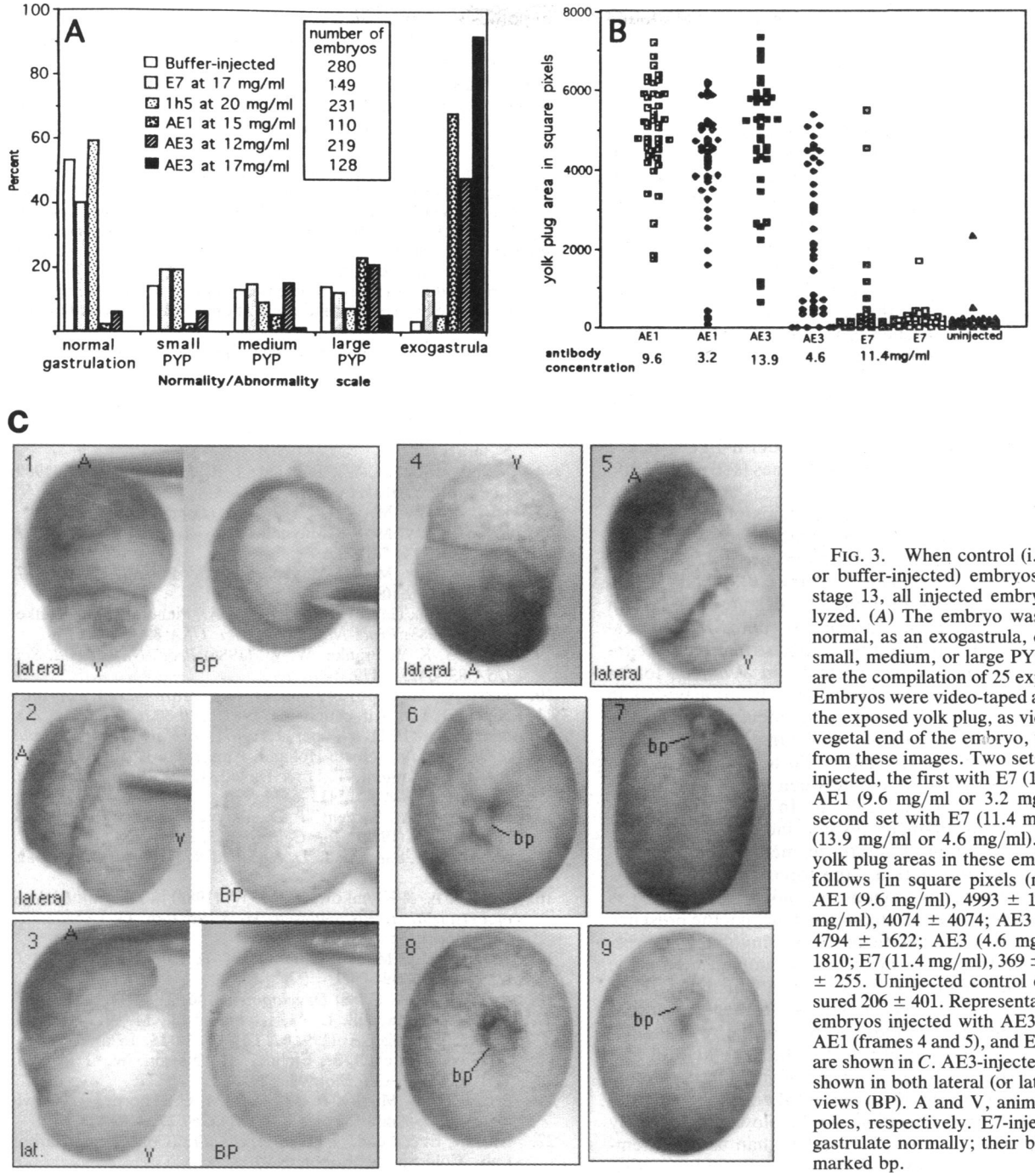

FIG. 3. When control (i.e., uninjected or buffer-injected) embryos had reached stage 13, all injected embryos were analyzed. (*A*) The embryo was classified as normal, as an exogastrula, or as having a small, medium, or large PYP. The results are the compilation of 25 experiments. (*B*) Embryos were video-taped and the area of the exposed yolk plug, as viewed from the vegetal end of the embryo, was measured from these images. Two sets of eggs were injected, the first with E7 (11.4 mg/ml) or AE1 (9.6 mg/ml or 3.2 mg/ml) and the second set with E7 (11.4 mg/ml) or AE3 (13.9 mg/ml or 4.6 mg/ml). The exposed yolk plug areas in these embryos were as follows [in square pixels (mean ± SD)]: AE1 (9.6 mg/ml), 4993 ± 1161; AE1 (3.2 mg/ml), 4074 ± 4074; AE3 (13.9 mg/ml), 4794 ± 1622; AE3 (4.6 mg/ml), 2189 ± 1810; E7 (11.4 mg/ml), 369 ± 1015 and 195 ± 255. Uninjected control embryos measured 206 ± 401. Representative images of embryos injected with AE3 (frames 1–3), AE1 (frames 4 and 5), and E7 (frames 6–9) are shown in *C*. AE3-injected embryos are shown in both lateral (or lat.) and vegetal views (BP). A and V, animal and vegetal poles, respectively. E7-injected embryos gastrulate normally; their blastopores are marked bp.

AE3 antibodies fail to bind to their epitopes in the superficial keratin filament system of the embryo. On the other hand, the nonuniform distribution of these antibodies in the deeper regions of the embryo (illustrated in Fig. 3) suggests that they can bind to, and presumably affect, the organization of the deeper keratin filament systems.

The pattern of keratin reactivity of AE1 and AE3 antibodies, both in *Xenopus* and in mammalian cells, suggests that these antibodies recognize spatially distinct epitopes. That two antibodies directed against the same cellular component produce a similar effect on developmental behavior is strong evidence that they exert their effect through interaction with that component and makes it less likely that their common effect on gastrulation (Fig. 3) is due to a fortuitous cross-reaction with an as yet unidentified nonkeratin polypeptide that is itself critical for normal gastrulation. Along these lines the gastrulation defect induced by the anti-IFA mAb (data not shown), although obscured by this antibody's nonspecific effects on cell division, appears similar to that seen in AE1/AE3-injected embryos and again suggests that these antibodies (i.e., AE1, AE3, and anti-IFA) disrupt gastrulation by binding to keratins.

The obvious question, then, is why does anti-keratin antibody 1h5 fail to disrupt gastrulation? The answer appears to lie in the differential accessibility of keratin epitopes in the living embryo. In contrast to AE1 and AE3, 1h5 appears to associate exclusively with the superficial keratin system; there is no apparent association of the injected 1h5 antibody with deeper structures (Fig. 1*C*). On the basis of the 1h5-induced disruption of the superficial keratin filament system (Fig. 1*F*) and its lack of an effect on gastrulation (Fig. 3*A*), we conclude that the integrity of the embryo's superficial keratin filament system is not required for normal gastrulation. Its role in the early embryo would, therefore, appear to be

similar to that of the dense keratin filament networks found in the epidermis of later stage tadpoles and adult frogs and in mammals—namely, to strengthen the organism's surface from mechanical damage originating from its environment (see ref. 5).

The *Xenopus* embryo must perform a number of complex and mechanically coupled cellular reorganizations to complete gastrulation. In the 4–5 h from the beginning to the end of gastrulation, the cell layers of the animal hemisphere thin through processes known as epiboly; the thin layer of cells is simultaneously pulled over the embryonic surface by convergent extension and, thereby, comes to form the embryonic surface (ectoderm) and the roof of the archenteron (endoderm). During this process, the yolk plug is pulled inward to form the floor of the archenteron and other endodermal tissues. In urodelian amphibians, these gastrulation movements depend critically on interactions between cells and the extracellular matrix; in contrast, in the anuran amphibian *Xenopus* gastrulation movements appear to be driven primarily by the rearrangement of subectodermal cells in the involuting and noninvoluting marginal zones (see refs. 25 and 26).

That the deeper keratin filament systems of the *Xenopus* embryo could play a role in the mechanical integration of the morphogenetic movements of *Xenopus* gastrulation is suggested by the observation, made by Perry (10), that urodelian amphibians do not have a substantial IF/desmosomal system until well after gastrulation. In contrast, both IFs and desmosomes are readily observed prior to gastrulation in *Xenopus*. For gastrulation to occur normally in *Xenopus*, forces generated within a specific region must be transmitted in specific directions. The deep keratin filament system and its associated desmosomal intercellular junctions effectively form a supracellular system that could act to increase the mechanical coupling within and between the morphogenetically active regions of the embryo. In the absence of a supracellular keratin filament system, induced by the antibody-induced disruption of keratin filaments or keratin filament/desmosomal interactions, the forces normally generated in specific embryonic regions may not be transmitted as effectively. In the urodelian amphibian embryo, the presence of other mechanisms, specifically those involving interactions with the extracellular matrix, may compensate for the absence of keratin filaments (6). *Xenopus*, which lacks such a mechanism, appears to rely on keratin filaments to ensure the mechanical integration required for normal gastrulation.

By assuming a mechanical role for the deep keratin filament system of the embryo, the speed of gastrulation should affect the severity of the observed anti-keratin-induced defects. The speed of gastrulation can be slowed ≈2-fold by maintaining embryos at 16–18°C, rather than at room temperature (23–24°C). Preliminary experiments indicate that embryos maintained at 16–18°C do exhibit less severe antibody-induced and spontaneous defects than those that gastrulate at room temperature (22–24°C; data not shown). It remains unclear, however, whether this rescue is due simply to the slowing of gastrulation or to some other temperature-dependent phenomena. In any case, our current working hypothesis is that anti-keratin antibodies AE1 and AE3 disrupt gastrulation by disrupting the integrity of the *Xenopus* embryo's deep keratin filament systems. We assume that these deep keratin filament systems act in an essentially passive manner, stabilizing cellular interactions and transmitting forces through morphogenically active tissues.

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